Superior Chemotherapeutic Efficacy of Amphotericin B in Tuftsin-bearing Liposomes against *Leishmania Donovani* Infection in Hamsters

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Chemotherapeutic efficacy of the amphotericin B (Amp B), which is the drug of choice for treatment of the leishmanial infections (kala-azar) that become resistant to the conventional chemotherapy using antimonials, has been examined in the *Leishmania donovani* infected hamsters after encapsulating the drug in tuftsin-free as well as tuftsin-bearing liposomes. The activity was significantly increased ($p < 0.05$) by delivering Amp B in tuftsin-free liposomes. This antileishmanial effect of the liposomized Amp B was further increased ($p < 0.05$) by grafting the natural macrophage-activator tetrapeptide, tuftsin (Thr–Lys–Pro–Arg), on the liposome's surface. This could possibly be attributed to both the enhanced drug tolerance after liposomization as well as to the increased uptake of tuftsin-bearing Amp B-laden liposomes by the macrophages. In addition to the increased efficacy, encapsulation of Amp B in the tuftsin-bearing liposomes also enhanced the drug accessibility to areas (e.g. bone marrow) that are otherwise inaccessible to the free drug. These results further demonstrate the usefulness of tuftsin-bearing liposomes as drug vehicles in treatment of the macrophage-based infections that have been reviewed recently (Agrawal, A.K. and Gupta, C.M. (2000). Tuftsin-bearing liposomes in treatment of macrophage-based infections, *Adv. Drug Deliv. Rev.*, 41, 135–146).

Keywords: Leishmania; Amphotericin B; Liposomes; Tuftsin; Hamsters

INTRODUCTION

The inherent tendency of "conventional" liposomes to concentrate in the mononuclear phagocyte system (MPS) has been exploited in enhancing the non-specific host defense against a variety of infections, and also as carriers of antibiotics in treatment of infections involving MPS (Patel, 1992; Ten Hagen *et al.*, 1998). As leishmania parasites mainly reside within the mononuclear phagocytic cells, a number of successful studies have been carried out by using liposomes as the antileishmanial drug carriers in treatment of the disease (Cauchetier *et al.*, 2000; Frezard *et al.*, 2000).

However, effective management of the kala-azar has proved to be difficult due to increasing resistance of the parasite to conventional anti-leishmanial drugs, like antimonials, and also due to its high toxicity (Coukell and Brogden, 1998; Yardley and Croft, 2000). Although the potent anti-fungal drug, Amp B is quite effective also as an anti-leishmanial agent, it suffers from several side effects including marked renal toxicity. The toxic effects of Amp B can be significantly minimized, while keeping intact the anti-leishmanial property, by encapsulating this drug in liposomes (Murray, 1999; Sundar 2001). The liposomized Amp B besides being effective against the *L. donovani* infections in the immunocompetent hosts has also been shown to be quite effective in T-cell deficient cases, which is quite unlike the other anti-leishmanial drugs, such as pentavalent antimony and pentamidine (Murray *et al.*, 1993). AmBisome (liposomal Amp B) was the first drug approved for the treatment of visceral leishmaniasis by the US FDA (Meyerhoff, 1999). As the host defense against this intracellular leishmanial infection is T-cell-dependent, kala-azar has predictably joined the list of AIDS-related opportunistic infections in endemic areas (Murray, 1999) and liposomal Amp B has also been shown to be quite

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effective in controlling the disease in such cases (Laguna *et al.*, 1999; Borrelli *et al.*, 2000; Mastroianni *et al.*, 2000).

We have previously demonstrated that encapsulation of stibanate in tuftsin-bearing liposomes markedly enhanced the efficacy of the drug (at least 200 times) against *L. donovani* infections in hamsters (Guru *et al.*, 1989). As an extension of this study, we have now examined the suitability of the tuftsin-bearing liposomes as Amp B vehicles in treatment of the *L. donovani* infections in hamsters.

MATERIALS AND METHODS

All the materials used were of the analytical grade. Egg phosphatidylcholine (egg PC) was isolated from egg yolk (Agrawal *et al.*, 1987). Cholesterol (CH) was obtained from Centron Research Laboratory (Mumbai, India) and was used after recrystalizing it three times from methanol. Amp B as Fungizone, a lyophilized pharmaceutical preparation in deoxycholate, was obtained from Sarabhai Chemicals (Baroda, India) and was reconstituted in 5% dextrose before use. Amicon Centriflo CF-25 was purchased from Millipore (Watford UK). RPMI-1640 and reagents for cell culture were from Gibco (Grand Island, USA).

Tuftsin

Tuftsin was modified at the C-terminus (Thr–Lys–Pro–Arg–NH–$(CH_2)_2$–NH–$COC_{15}H_{31}$) to facilitate its incorporation in the liposomes bilayer. Both tuftsin and modified tuftsin were prepared as described earlier (Singhal *et al.*, 1984).

Animals

Male golden hamsters (*Mesocricetus auratus*) were used for *in vivo* evaluation of the liposomized Amp B preparations, while BALB/C mice (weight 18–22 g) were used for isolating macrophages for the *in vitro* experiments.

Parasites

The *Leishmania donovani* strain (HOM/IN/80/Dd 8), originally isolated from a kala-azar patient from Bihar (India), was a kind gift from professor P.C.C. Garnham, Imperial College, London. The strain is being regularly maintained *in vitro* as promastigotes in NNN medium or in Leibovitz (L-15) medium supplemented with 10% fetal tryptose phosphate broath (TPB) and 10% fetal bovine serum (FBS), and as amastigotes in golden hamsters.

Infection

In Vivo

Amastigotes were isolated from the spleen of infected hamsters (>1 month old infection). These were suspended in Locke's solution (8.0 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.3 g KH_2PO_4 and 2.5 g glucose in 1 l, pH 7.2) to give about 10^8 amastigotes/ml. One hundred microlitre of this suspension (containing about 10^7 amastigotes) was administered intracardiacly (i.c.) to each hamster (weight 40–45 g). The establishment of infection was assessed by sacrificing 3–4 animals and measuring the parasites loads in their spleens.

In Vitro

Peritoneal macrophages were obtained from BALB/C mice 3–4 days after intraperitoneal injection of 2% starch solution (0.5 ml). The macrophages were harvested in RPMI-1640 medium containing heparin. These were washed twice and resuspended in complete RPMI-1640 medium supplemented with 10% fetal calf serum. Gentamycin (40 μg/ml) was added to the medium to prevent bacterial growth. Cells were plated in multi well slide Lab Tek Chambers (Miles, USA) and incubated for 24 h at 37°C. Non-adherent cells were removed by thorough washing. The cells were routinely found to be more than 95% viable by trypan blue exclusion test. Promastigotes at a ratio of three parasites per macrophage were used to infect the cultures. Infection was permitted to proceed for 4 h at 37°C, after which all the extracelluar organisms that failed to parasitize the macrophages were removed by washing. In most instances 75–80% of the macrophages were to be infected. The infected macrophages were incubated in the culture medium under 5% CO_2 atmosphere for 24 h prior to drug treatment.

Liposomes

Liposomes were prepared from egg phosphatidylcholine and cholesterol (molar ratio 7:3) by probe sonication and fractionated by centrifugation as described earlier (Agrawal *et al.*, 2001). Briefly, the lipids (total weight 45 mg), with or without tuftsin (7–8% by weight of PC), were dissolved in chloroform in a round-bottomed flask and to it Amp B (1 mg) was added in methanol. The organic solvents were removed under reduced pressure, resulting in the formation of a thin lipid film on the wall of the flask. The dried lipid mixture was dispersed in 150 mM saline and sonicated for 30 min under nitrogen atmosphere using a probe-type sonicator. It was centrifuged at 12,000*g* for 30 min at 4°C. The supernatant was dialyzed against 150 mM saline to remove free Amp B, and the amount of Amp B incorporated in liposomes was determined spectrophotometrically (Mehta *et al.*, 1986). Circa 85% of the Amp B was found to be associated with the liposomes. The outer diameter of the liposomes, as determined by

electron microscopy, was circa 80 nm (Agrawal and Gupta, 2000b).

Treatment

In Vivo

Hamsters on day 15–18 post infection were divided into four groups, each group consisting of a large number of animals with 4–5 animals for each test dose. Animals in Group-I and Group-II were given free Amp B and Amp B in tuftsin-free liposomes (Lip-Amp B), respectively, while Group III was given Amp B in tuftsin-bearing liposomes (Tuft-Lip-Amp B). The Group IV was treated as controls and was given saline only. The animals were sacrificed 15 days after the treatment. Their spleen impression smears were prepared and stained with Giemsa to assess the amastigote counts. Inhibition of the amastigote number was calculated using the following equation:

$$\text{PI } 100 - (\text{NT}/\text{NC} \times 100)$$

where PI, percentage inhibition; NT, actual number of amastigotes/100 spleen cell nuclei after the treatment; NC, mean number of amastigotes/100 spleen cell nuclei in control animals on the corresponding day of the autopsy of treated animals.

In Vitro (Treatment of Infected Macrophages)

Pulse treatment was used in these experiments (Rabinovitch *et al.*, 1986). Infected macrophages were washed once with the medium and placed in complete RPMI-1640 medium containing free Amp B, Lip-Amp B or Tuft-lip-Amp B. The mixture was incubated for 3 h at 37°C under 5% CO_2 atmosphere. It was washed and the complete medium replaced with the fresh medium without any drug, and then incubated for 20 h in a CO_2 incubator. At the end of this period, the cells were fixed in methanol and stained with Giemsa. The number of amastigotes/100 macrophage nuclei in drug treated and control cultures were determined. The percent of leishmanicidal activity was calculated according to the following equation:

$$\text{PI } 100 - (\text{NT}/\text{NC} \times 100)$$

where PI, percentage inhibition; NT, number of amastigotes/100 nuclei in treated group; NC, number of amastigotes/100 nuclei in control group.

RESULTS

The effect of tuftsin mediated liposome targeting to macrophages on antileishmanial activity of Lip-Amp B was assessed by evaluating the efficacies of Lip-Amp B and Tuft-Lip-Amp B against *L. donovani* infections *in vitro*. We have previously demonstrated that Amp B incorporated in egg PC/CH appeared to be much more effective as compared to that in Soya PC/CH liposomes (Ahmad *et al.*, 1991). Therefore, the former formulation was used throughout the study. Table I shows that anti-leishmanial activities of both free and liposomized Amp B increased with increasing Amp B concentration in the medium. While no problems were encountered by using Amp B up to 10 μg/ml concentration in liposomes, extensive cell lysis occurred when the drug was used at this concentration in native form. The anti-leishmanial activity of Lip-Amp B at all Amp B concentration was higher than that observed with free Amp B in identical conditions (Table I). This activity further increased when tuftsin was grafted on the surface of Amp B containing liposomes. The difference observed between the anti-leishmanial activities of Lip-Amp B and Tuft-Lip-Amp B was highly significant ($p < 0.01$) at Amp B concentrations lower than 10 μg/ml. These results clearly indicate that encapsulation of Amp B in liposomes helps not only in reducing the drug toxicity but also in increasing the drug efficacy against *L. donovani* infections *in vitro*. Thus, to achieve 50% inhibition in *L. donovani* growth and multiplication only 5 and 2.5 μg/ml concentrations of Amp B were required after its encapsulation in tuftsin-free and tuftsin bearing liposomes, respectively.

To examine the validity of the above results under *in vivo* conditions, we have evaluated the anti-leishmanial activity of various Amp B preparations in *L. donovani*-infected hamsters. The anti-leishmanial activity was assessed by measuring the parasite load in spleens of infected animals after their treatment with various formulations of Amp B on day 15 post infection. The results given in Table II indicate that the anti-leishmanial activity of Lip-Amp B was significantly ($p < 0.05$) greater than that observed with free Amp B. This difference in the activity further increased ($p < 0.01$) by grafting tuftsin on Lip-Amp B surface.

The tissue distribution experiments showed the higher and faster uptake of Tuft-Lip-Amp B from the circulation with most of the liposomes being cleared from the circulation within 1 h after administration as compared to Lip-Amp B. Moreover, the drug distribution in various tissues was also influenced by the degree of infection and

TABLE I Enhanced effect of tuftsin-bearing Amp B laden liposomes on *L. donovani* infections in mouse macrophages *in vitro*. (Value shown are means of 3–4 experiments ± S.D)

Sample	Amp B dose μg/ml)	%inhibition of infection
Amp B	1.25	18.26 ± 1.92
	2.5	29.61 ± 3.37
	5	39.62 ± 3.99
	10	Cell lysis
Lip-Amp B	1.25	39.10 ± 2.11
	2.5	40.39 ± 8.36
	5	51.89 ± 2.02
	10	69.56 ± 1.24
Tuft-Lip-Amp B	1.25	46.19 ± 0.96
	2.5	53.54 ± 3.76
	5	61.40 ± 4.65
	10	72.90 ± 3.84

TABLE II Superior chemotherapeutic efficacy of tuftsin-bearing Amp B laden liposomes on *L. donovani* infections in hamsters. (Each experiment was carried out at least two times. Values are means of 3–5 animals (S.D)

Sample	Amp B dose (single; mg/kg)	% inhibition of infection in spleen
Amp B	0.5	37.6 ± 7.8
		38.7 ± 7.0
	1	38.5 ± 27.3
		37.2 ± 6.8
Lip-Amp B	0.5	60.1 ± 7.9
		59.9 ± 7.1
	1	71.2 ± 5.7
		68.7 ± 4.1
Tuft-Lip-Amp B	0.5	81.4 ± 7.3
		75.0 ± 9.2
	1	78.8 ± 5.3
		80.9 ± 6.7

the drug concentration, and in the case of Tuft-Lip-Amp B was maximum for all organs of infected animals except kidney at 1 h, suggesting immediate uptake of these liposomes by the macrophage-rich organs (liver, spleen and lung). However, this was not the case with Lip-Amp B, indicating that, mere liposomization of Amp B is not enough to significantly alter its distribution in the macrophage-rich organs of the infected animals (Agrawal *et al.*; manuscript under preparation).

These results clearly demonstrate that the anti-leishmanial activity of Lip-Amp B is significantly increased by grafting the macrophage activator tetrapeptide, tuftsin, on the liposomes surface. Initial experiments with multiple doses of free Amp B, Lip-Amp B and Tuft-Lip-Amp B administered to the infected hamsters produced significantly high degree of inhibition in spleen for both Lip-Amp B and Tuft-Lip-Amp B, as compared to that treated with free Amp B only. However, under these conditions, no statistical difference was seen between the anti-leishmanail activities of Lip-Amp B and Tuft-Lip-Amp B (Agrawal *et al.*; unpublished observations).

DISCUSSION

Leishmaniasis has re-emerged from near eradication. The estimate for the incidence and prevalence of the disease world-wide is 0.5 and 2.5 million per year, respectively. Numerous efforts have been made towards the development of effective vaccines against leishmaniasis. In spite of promising results with Phase 3 trials with the first generation vaccine (killed leishmania organism mixed with low concentration of BCG as an adjuvent), there is no effective leishmania vaccine as yet on the horizon, and chemotherapy remains the only practical way to combat all forms of the disease. The major front line drugs available for leishmaniasis are toxic. Several doses need to be given over a prolonged period of time and development of drug resistance becoming a serious problem.

We have previously demonstrated that the efficacy of Tuft-Lip-Amp B was markedly enhanced as compared to Lip-Amp B against *A. fumigatus* infections in mice, both in terms of the survival times of animals and reduction of fungal load, primarily due to macrophage activation by tuftsin. Tuftsin incorporation in the Lip-Amp B did not seem to affect the drug toxicity, as we observed almost identical LD_{50} value for both Lip-Amp B and Tuft-Lip-Amp B (Owais *et al.*, 1993).

In our earlier biodistribution study, we showed that increase in the activity of Lip-Amp B against leishmaniasis was related to the reduced drug toxicity rather than altered drug distribution at the site of infection (Ahmad *et al.*, 1991). In the present study, we show that uptake of Tuft-Lip-Amp B is higher and faster from the circulation as compared to Lip-Amp B. Also, grafting of tuftsin on Lip-Amp B alters significantly its distribution in the macrophage-rich organs of infected animals. It may thus be suggested that the higher efficacy of Tuft-Lip-Amp B observed here could be due to both the increased drug tolerance and enhanced uptake of liposomized drug by the macrophage-rich organs of the infected mice. Tuftsin-bearing liposomes also increase the drug accessibility to areas like bone marrow which are otherwise inaccessible to free Amp B, and are the main cause of relapse of the leishmanial infection. The improvement in the therapeutic efficacy of the Tuft-Lip-Amp B might have resulted from the respiratory burst-inducing activity of tuftsin (Singh *et al.*, 1992), in addition to the effect of targeted drug delivery of the drug to the macrophages.

In conclusion, the present study clearly shows that the encapsulation of Amp B in liposomes significantly enhances its anti-leishmanial activity, which is consistent with other reports (Bodhe *et al.*, 1999; Mastroianni *et al.*, 2000). Also, it demonstrates that tuftsin-mediated targeting of Amp B loaded liposomes to macrophages further increases the drug efficacy. Moreover, as observed *in vitro*, the difference between the anti-leishmanial activities of Lip-Amp B and Tuft-Lip-Amp B (Table II) was much more prominent at lower Amp B dose (0.5 mg/kg), as compared to higher dose (1.0 mg/kg). Besides, reducing the parasite load in the spleen, this formulation was quite effective in eliminating the liver and bone marrow parasite loads (Agrawal *et al.*; unpublished observations). This could perhaps be attributed to the higher uptake of Tuft-Lip-Amp B, as compared to Lip-Amp B, by the monocytes/macrophages (Singhal *et al.*, 1984), which in turn should help the MPS cells in achieving an optimal intracellular drug concentration. Multiple doses of Lip-Amp B and Tuft-Lip-Amp B to the infected hamsters produced significantly high degree of inhibition in spleen, as compared to that treated with free Amp B only. However, no statistical difference was seen between the anti-leishmanail activities of Lip-Amp B and Tuft-Lip-Amp B (Agrawal *et al.*; unpublished observations). This could possibly be attributed to the saturation of tuftsin receptors on monocytes/macrophages due to its high dose as tuftsin has been shown to have inhibitory

effects at its high concentration (Babcock *et al.*, 1983). This study is in accordance with our previous study (Guru *et al.*, 1989), which showed an increased anti-leishmanial activity of liposomized sodium stibogluconate after grafting tuftsin on the liposomes surface.

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